PRODUCTION OF THE LIPIDATED FORM OF THE PEPTIDOGLYCAN-ASSOCIATED LIPOPROTEINS OF GRAM-NEGATIVE BACTERIA

Field Of The Invention

This invention is directed to the expression of the lipidated form of the peptidoglycan-associated protein of gram-negative bacteria and the use of that recombinant lipidated protein in antigenic compositions.

Background Of The Invention

The cell walls of gram-negative bacteria contain cross-linked moieties known as peptidoglycans. A number of gram-negative bacteria produce proteins which are covalently linked to the peptidoglycans. Such a protein is referred to as a peptidoglycan-associated lipoprotein (PAL). PALs are present as part of the tol locus in a number of gram-negative bacteria, including Legionella pneumophila (Bibliography entry 1), Escherichia coli (2), Haemophilus ducreyi (3), Campylobacter jejuni (4), Pseudomonas putida (5), Brucella abortus (6), Pseudomonas aeruginosa, Klebsiella aerogenes, Serratia marcescens, Proteus vulgaris, Salmonella typhimurium (7) Actinobacillus pleuropneumoniae (8), Helicobacter pylori (9) Chlamydia pneumoniae (10), and Chlamydia trachomatis (11).

Other PAL-containing bacteria are the Haemophilus influenzae (H. influenzae) bacteria. The H. influenzae bacteria are divided into two groups. Those strains which possess a known capsule are typed by the serological reaction of the capsule with reference antisera. Types a-f have been identified. Strains which fail to react with any of the reference

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antisera are known as nontypable.

H. influenzae type b (Hib) is the most
frequent cause of neonatal meningitis and other
invasive infections in the United States (12). The
major incidence of childhood meningitis occurs between
the ages of one and five years. Sixty percent of those
meningitis cases due to Hib occur in children under the
age of two (12).

Nontypable H. influenzae (NTHi) is a gram-
negative organism which causes a number of diseases,
including pneumonia, bacteremia, meningitis, postpartum
sepsis, and acute febrile tracheobronchitis in adults
(13). NTHi has been reported to cause between 20 and
40 percent of all cases of otitis media seen in young
children (14,15,16). Children may experience multiple
infections due to the same organism since infection
confers no long lasting immunity. Current therapy for
chronic or repeated occurrences of otitis media
includes administration of antibiotics and insertion of
tubes to drain the inner ear. NTHi strains have also
been implicated as a primary cause of sinusitis (17).
Additionally, NTHi causes neonatal sepsis.

Current capsular-based antigenic compositions
are ineffective against NTHi. The surface of these
bacteria has been shown to be extremely antigenically
variable, with the major outer membrane proteins, P1
and P2, being particularly diverse (18,19). In humans,
the presence of serum bactericidal antibodies has been
reported to correlate with protection from otitis media
caused by sensitive NTHi strains (20).

Candidates for inclusion in antigenic
compositions against NTHi should be highly conserved at
the amino acid level, surface exposed (in particular,
outer membrane proteins), elicit bactericidal
antibodies, and be present in all isolates. Previous

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research has shown that the P6 (also known as PBOMP-1
and HiPAL) (21) protein of NTHi meets all of these
criteria. The purified native proteins have been shown
to elicit bactericidal antibodies (22,23,24,25) and are
conserved antigenically (22,23,26,27).

Evaluation of the genetic sequence of the P6
gene has shown that it is highly conserved among otic
NTHi isolates and thus the protein sequence is also
highly conserved. Native P6 is a lipoprotein, more
specifically a PAL, which is modified at the amino-
terminal cysteine with lipids. This protein is present
in *H. influenzae* in relatively small amounts (less than
1% of total outer membrane proteins), making
purification from the native organism of useful
quantities quite difficult. Thus, a recombinant
version of P6 is required for further development as a
component in antigenic compositions.

Several laboratories were unable to express
lipidated rP6 in large quantities in *E. coli* (28,29).
As a result, initial recombinant constructs expressing
P6 in *E. coli* could express only a nonlipidated version
of the protein. These groups reported that, while the
lipidated P6 protein purified from *H. influenzae* was
more immunogenic than nonlipidated rP6 purified from *E.
coli*, it was difficult to engineer a DNA vector which
would express lipidated P6 (28,29); i.e., not better
than the low levels of native P6 expressed by *H.
influenzae*.

Previous attempts to express lipidated rP6
relied on promoters which were not under tight
transcriptional regulation, such as trc, taq, lac and
P_L-C1857. It was theorized that this somewhat leaky
transcription led to subtle effects on the *E. coli*
which contributed to low levels of expression of the
lipidated protein. Experimental evidence indicated

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that the ability of signal peptidase II to add lipid to the N-terminus of the protein was not responsible for the low yield of processed P6 (data not shown).

While the P6 protein of *H. influenzae* has been a primary candidate for inclusion in antigenic compositions against Haemophilus disease (20,23,24,25,30,31), the relatively small amounts available from *H. influenzae* have made recombinant expression of this protein essential. Previous efforts to express lipidated P6 protein in meaningful quantities have been unsuccessful (28,29). Thus, researchers have focused on the expression and purification of multiple forms of nonlipidated P6.

The antibody response engendered by the nonlipidated rP6 was biologically functional, capable of protecting infant rats from meningitis (28) and eliciting bactericidal antibodies (28,29), but of a lower magnitude than those elicited by lipidated native P6 (28).

Therefore, there is a need to construct host cell-expression vector systems which express lipidated PALs of gram-negative bacteria. In particular, there is a need to construct host-cell expression vector systems which express lipidated rP6, which can then be included in antigenic compositions against *H. influenzae*.

Summary Of The Invention

Thus, it is an object of this invention to develop genetic constructs capable of expressing lipidated PALs of gram-negative bacteria in bacterial host cells.

It is a particular object of this invention to develop genetic constructs capable of expressing

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lipidated rP6 in bacterial host cells, in particular, in *E. coli*.

It is a further object of this invention to include that lipidated rP6 in antigenic compositions for administration to a mammal to prevent disease caused by *H. influenzae*.

These and other objects of the invention as discussed below are achieved by the cloning and expression in bacterial cells of the lipidated forms of PALs through the use of tightly regulated promoters in the expression vectors.

The invention is exemplified by the cloning and expression in bacterial cells of the lipidated form of recombinant *H. influenzae* P6 protein through the use of such promoters in the expression vector.

Specifically, for the expression of lipidated recombinant P6, plasmids are constructed which contain an arabinose inducible promoter or a T7 promoter, wherein the promoter is operatively linked to an isolated and purified DNA sequence comprising a DNA sequence which encodes the P6 protein, and wherein the DNA sequence, under the control of said promoter, is expressed in lipidated form.

In turn, a bacterial host cell is transformed, transduced or transfected with such a plasmid and is then cultured under conditions which permit the expression of the lipidated rP6 by the host cell.

In another embodiment of this invention, the lipidated rP6 is used as an immunogen in antigenic compositions against all pathogenic *H. influenzae*, including both type b and nontypable *H. influenzae*.

When purified, the recombinant protein is indicated to be lipidated by several criteria and, most importantly, is much more immunogenic than the non-

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lipidated form of P6 previously used.

The lipid modification of the amino-terminal cysteine by signal peptidase II has been shown to make proteins more immunogenic than their non-lipidated forms (28,29,32). These forms have been evaluated for immunogenicity and antigenic relatedness to lipidated P6. All have shown decreased immunogenicity as compared to the native lipidated protein.

This allows much lower doses to be used to immunize humans and thus makes the lipidated rP6 a more commercially viable candidate for inclusion in antigenic compositions. These increased titers allow the use of lower P6 protein doses in humans, which would provide a cost savings in the production of that protein.

The isolated and purified lipidated rP6 protein is used to prepare an antigenic composition which elicits a protective immune response in a mammalian host. The antigenic composition may further comprise one or more of an adjuvant, diluent or carrier. Examples of such adjuvants include aluminum hydroxide, aluminum phosphate, Stimulon™ QS-21, MPL™, IL-12 and cholera toxin. The antigenic composition is administered to a mammalian host in an immunogenic amount sufficient to protect the host against disease caused by *H. influenzae*.

Brief Description Of the Figures

Figure 1 depicts the cloning of the *pal* gene encoding the P6 protein with the native lipoprotein signal peptide by PCR amplification from the chromosome of nontypable *H. influenzae* strain P860295.

Figure 2 depicts the homogeneity and identity of lipidated rP6. Fifteen percent SDS-PAGE gels were

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loaded with samples containing approximately 10μg of
lipidated rP6 in the initial extract (lane 1) and the
pool of anion exchange purified lipidated rP6 (lane 2).
Lanes labeled S contain prestained low molecular weight
standards from Bio-Rad. Figure 2A is the Coomassie
stained gel. Figure 2B is the western immunoblot of a
similar gel.

Figure 3 depicts the SDS-PAGE analysis of
fractions from the purification of lipidated rP6.
Aliquots of steps in the purification process of
lipidated rP6 were electrophoresed on a 4-20% gradient
gel system. Lanes: 1, Permeate from diafiltration with
lysis buffer; 2, Permeate from diafiltration with
Triton™ X-100; 3, Permeate from diafiltration with
Tris™ buffer; 4, Permeate from diafiltration with
Zwittergent™ 3-14; 5, Permeate from diafiltration with
Zwittergent™ 3-14/0.5 M NaCl; 6, Permeate from
diafiltration with Tris™ buffer; 7, Permeate from
diafiltration with sarcosyl; 8, Mark 12 Standard; 9,
Permeate from diafiltration with Tris™ buffer; 10,
Permeate from diafiltration with Zwittergent™ 3-12 at
room temperature; 11, Permeate from diafiltration with
Tris™ buffer; 12, Permeate from concentration step; 13,
Permeate from diafiltration with Zwittergent™ 3-12 at
55°C; 14, Permeate from diafiltration with Tris™ buffer
at 55°C; 15, Permeate from diafiltration with
Zwittergent™ 3-12 at 55°C.

Detailed Description Of The Invention

In order to overcome the recognized
difficulty in expressing usable amounts of lipidated
PALs, such as lipidated rP6, a strategy was devised
involving the use of tightly regulated promoters and

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host strains devoid of cytoplasmic and perplasmic
proteases.

Previous unsuccessful efforts to express
lipidated P6 protein in meaningful quantities for
commercial use all relied on changing the promoter
sequence and/or making changes in the signal sequence
recognized by signal peptidase II.

As discussed below, plasmids were constructed
containing the *pal* gene encoding P6 under the control
of the T7 promoter (plasmid pPX4019 – Example 1) and
the arabinose inducible promoter (plasmid pPX4020 –
Example 2). Exemplary bacterial strains and media are
described in Example 3. Both pPX4019 and pPX4020
express lipidated P6 protein in the *E. coli* strains
tested, as determined by western blot analysis with P6-
specific monoclonal antibodies, sizing on SDS-PAGE gels
which indicated a lack of a signal sequence and visual
observation of expression levels in Coomassie stained
gels (see Example 4 and Figures).

Plasmid pPX4020 produced increased levels of
rP6 protein expression in the *E. coli* strains BL21 and
BLR, with the highest levels in strain BLR. Therefore,
plasmid pPX4020 was chosen for further studies. Growth
of larger quantities of lipidated rP6 expressing *E.
coli* was performed in host strain BLR. All subsequent
experiments utilized this host-vector system.

The plasmid construct described herein as a
preferred embodiment (pPX4020) uses the arabinose
inducible promoter system which has several unique
features: It is tightly regulated and almost
completely inactive if no arabinose is present and some
glucose is present. It is also modulatable in that it
shows increasing induction levels as increasing
arabinose is added to the culture medium. These
factors in combination with the BLR strain of *E. coli,*

which is highly protease deficient and recombination
deficient, allow significant expression of the
lipidated P6 protein.

     Batch scale purification of lipidated rP6
involves differential centrifugation, differential
detergent extraction and anion exchange chromatography
(see Example 4).

     However, to become a viable candidate for
inclusion in an antigenic composition, the expressed
lipidated rP6 must be purified by a method which is
amenable to large scale. Diafiltration is a method
suitable to large scale purification. The
diafiltration process for extracting lipidated rP6 is
complicated because lipidated rP6 is tightly associated
with peptidoglycans.

     As described in Example 5, solubilization of
the lipidated rP6 was accomplished following
differential detergent extraction much like the native
protein obtained from *Haemophilus* (Hi-P6) (33), but
with tangential flow diafiltration used instead of
centrifugation. Detergents such as dodecylmaltoside,
deoxycholate, Zwittergent™ 3-08, 3-12, and 3-14 were
all tested and found to be acceptable to extract the
lipidated rP6 when used in the 0.2-1%(w/v) range. The
lipidated rP6 could also be solubilized in a sodium
borate buffer, pH 9.5 at 65°C. The flexibility of
detergent choice permitted the use of Zwittergent™ 3-12
to extract the lipidated rP6. The choice of
Zwittergent™ 3-12 thus minimizes the number of
components required to produce a multi-component
antigenic composition. Whereas the native Hi P6 is
obtained in essentially pure form following
solubilization, the recombinant protein is solubilized
along with several *E. coli* proteins. The relative
amount of these proteins can be varied and in some

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cases nearly eliminated by the choice of detergent used
in the final extract. The lipidated rP6 is separated
from any remaining *E. coli* proteins by anion exchange
chromatography (see Example 6).

5   This method of extracting PALs combines the
clarification and extraction processes into one unit
operation. The product is extracted from the cells and
it is separated from cell debris with only one
continuous diafiltration process. In addition, the
10   PALs are extracted in a semi-purified state which
simplifies the downstream processing steps. Finally,
this process is very scalable, because the only
requirement is that the surface area of the membranes
be increased proportionally with the amount of cells.
15   This extraction process avoids the use of
centrifugation, a method which is not preferred for use
in large scale extraction. After extraction, the
lipidated rP6 is purified by conventional techniques.
              Analysis of the lipidated rP6 was consistent
20   with the characterization of the recombinant protein as
a lipoprotein, as expected. The molecular size as
determined by MALDI-TOF mass spectrometry shows that
the purified recombinant protein is larger than
expected from its amino acid sequence alone (see
25   Example 7). The size of the protein, combined with the
amino acid analysis detailed in Table 1 (see Example
8), indicate that the signal sequence has been removed
and the protein is in the mature form. The existence
of a blocked amino-terminus, as demonstrated by amino-
30   terminal amino acid analysis (see Example 9), also
shows that modification of the terminal cysteine
residue has taken place. Taken together, these results
show that the signal sequence, which has been shown to
be recognized and processed by *E. coli* resulting in
35   lipidated P6 (23), has been removed and that the

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recombinant P6 purified here is lipidated at its amino-
terminus.

Previous investigators have demonstrated that
antibody levels against P6 after naturally occurring
NTHi infections have an inverse correlation with
disease incidence (34,35,36), making the production of
high titers against P6 a goal of any immunization
program using this antigen. The native lipidated P6
protein is present in very small quantities (less than
1% of the outer membrane proteins) (33), which makes
purification of commercially viable quantities
problematic at best.

A critical advantage of the lipid-modified
rP6 over the previously expressed non-lipidated rP6 is
the enhanced immunogenicity associated with the lipid
modification. Two reports have shown that, while non-
lipidated rP6 is capable of eliciting biologically
active antibodies, it is less immunogenic than the
native lipidated protein (28,29).

In contrast, the animal immunogenicity data
presented in Tables 2-4 below (see Examples 10 and 11)
show that the lipid modification of recombinant P6 also
increases the immunogenicity of this antigen in the
mouse model. The up to a 2-log increase in geometric
mean antibody titers (GMT) at week 6 is quite
significant and makes the lipidated form of the rP6
protein a practical candidate for inclusion in
antigenic compositions. This is bolstered by the
results in these experiments that lipidated rP6 did not
interfere with the immune response generated by the
other antigens tested.

Taken together, these data support the view
that lipidated rP6 is viable for inclusion in antigenic
compositions against *H. influenzae*. Although
exemplified below with lipidated rP6 from NTHi,

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lipidated rP6 from Hib is also suitable for inclusion
in the antigenic compositions of this invention.

A variety of bacterial host cell-vector
systems are suitable for use to express the lipidated
rP6 protein used in the antigenic compositions of this
invention in addition to those detailed in Examples 1-
3.

These expression systems place the gene
encoding the recombinant lipidated PAL under the
control of a tightly regulated promoter. Under
specific conditions, these promoters operate to down
regulate the production of the recombinant PAL mRNA,
and consequently mitigate any detrimental effects on
the host cell due to the production of the recombinant
lipidated PAL (37). This tight regulation can then be
removed under specific conditions to allow for
maximized recombinant lipidated PAL expression in the
host cell.

These tightly regulated promoters (which may
be together with other control elements) include, but
are not limited to, the arabinose inducible promoter
(38), the T7 promoter which may be modified to be under
control by nutL/N antitermination function (39,40) or
by Mu C (41), the P_L promoter in combination with
antiterminator (42), the SP6 RNA polymerase and SP6
promoters (43), the colicin promoter (44), the tetA
promoter/operator (45), the rhamnose and phosphate
promoters (46), the LacR/O, tetR/O and AraCI1-12
regulatory elements (47), and invertible promoters
(48).

The vector system is compatible with the host
cell used. Suitable host cells include bacteria
transformed, transfected or transduced by conventional
techniques with plasmid DNA, cosmid DNA or
bacteriophage DNA. Examples of bacterial hosts include

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E. coli, B. subtilis, Salmonella and *Shigella*.

To construct such a vector, the *pal* DNA is inserted into a plasmid vector containing a promoter under tight transcriptional control, and other control elements are ligated into specific sites within the vector, so that when the plasmid vector is inserted into a bacterial host cell, the *pal* DNA can be expressed by the host cell.

The plasmid is introduced into the host cell by transformation, transduction or transfection, depending on the host cell-vector system used. The host cell is then cultured under conditions which permit expression of the lipidated rP6 protein by the host cell. A host cell containing a plasmid with the arabinose inducible promoter is induced with L-arabinose, while a host cell containing a plasmid with the T7 promoter is induced with IPTG.

The lipidated PALs are useful in the preparation of antigenic compositions to confer protection to mammals against diseases caused by the corresponding bacteria. For example, the lipidated rP6 protein is useful in the preparation of antigenic compositions to confer protection to mammals against diseases caused by *H. influenzae*.

These antigenic compositions comprise an isolated and purified lipidated PALs, such as lipidated rP6 protein, wherein the antigenic composition elicits a protective immune response in a mammalian host. Multivalent antigenic compositions are provided by including other proteins, such as by combining the lipidated rP6 with the UspA2 protein of *Moraxella catarrhalis* (which is described in PCT International Application Number WO 98/28333 (49), which is hereby incorporated by reference), a causative agent of bacterial otitis media, and the recombinant lipidated

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rP4 protein of *H. influenzae* (also known as protein
"e") (which is described in United States Patent Number
5,601,831 (50), which is hereby incorporated by
reference).

Antigenic compositions containing a lipidated
PAL, such as lipidated rP6 protein, may be mixed with
immunologically acceptable diluents or carriers in a
conventional manner to prepare injectable liquid
solutions or suspensions. Such diluents or carriers
include, but are not limited to, PBS, physiologic
saline, buffered isotonic solutions, liposomes and
ISCOMS. The level of antibodies elicited by the
antigenic compositions may be improved by using certain
adjuvants such as aluminum hydroxide, aluminum
phosphate, Stimulon™ QS-21 (Aquila Biopharmaceuticals,
Inc., Framingham, MA), MPL™ (3-O-deacylated
monophosphoryl lipid A; RIBI ImmunoChem Research, Inc.,
Hamilton, MT), IL-12 (Genetics Institute, Cambridge,
MA), the heat-labile toxin of *E. coli*, and cholera
toxin (either in a wild-type or mutant form, for
example wherein the glutamic acid at amino acid
position 29 is replaced by another amino acid,
preferably a histidine, in accordance with PCT
International Application Number WO 00/18434) (51).

The antigenic compositions of this invention
are administered by injection in a conventional manner,
such as subcutaneous, intradermal or intramuscular
injection into humans, as well as by oral, mucosal,
intranasal or vaginal administration, to induce an
active immune response for protection against disease
caused by a gram-negative bacterium, such as *H.
influenzae*. The dosage to be administered is
determined by means known to those skilled in the art.
Protection may be conferred by a single dose of the
antigenic compositions, or may require the

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administration of several doses, in addition to booster
doses at later times to maintain protection.

    In order that this invention may be better
understood, the following examples are set forth. The
examples are for the purpose of illustration only and
are not to be construed as limiting the scope of the
invention.

<u>Examples</u>

    Standard molecular biology techniques are
utilized according to the protocols described in
Sambrook et al. (52).

<u>Example 1</u>
Construction of Plasmid pPX4019 Containing
<u>the pal Gene and the T7 Promoter</u>

    As depicted in Figure 1, the pal gene
encoding the P6 protein was cloned with the native
lipoprotein signal peptide by PCR amplification from
the chromosome of non-typable $H.$ $influenzae$ strain
P860295. Using mutagenic primers which created an NdeI
restriction site encompassing the start codon at the 5'
end of the gene (GGAGAAAT<u>CATATG</u>AACAAATTTG)(SEQ ID NO:1)
and a $Hind$III site in the region 3' of the stop codon
(GGATCCTGTTTTTC<u>AAGCTT</u>AGAAATACTAAG)(SEQ ID NO:2), the
PCR fragment containing the pal gene was cloned into
the pCRII expression vector (Invitrogen, Carlsbad, CA)
and screened by restriction analysis. The resulting
plasmid was used as the source for the NdeI/$Hind$III
fragment containing the pal gene for P6 which was
cloned into the NdeI and $Hind$III sites of expression
vector pET27b (Novagen, Madison, WI). The design of
this construction places the gene for P6 under the

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control of the T7 promoter and takes advantage of a
consensus ribosome binding site in the vector. The
initial clones were identified in a non-permissive *E.
coli* host (DH5α) by restriction analysis. A single
plasmid isolate was chosen and saved as pPX4019. This
plasmid was used to transform the permissive host
strain BL21(DE3,pLysS) (Novagen) for expression
studies.

Example 2
Construction of Plasmid pPX4020 Containing
the *pal* Gene and the Arabinose Inducible Promoter

A second P6 protein expression plasmid was
constructed placing the *pal* gene under the control of
the tightly regulated arabinose inducible promoter
(38). This plasmid was generated by subcloning the
*Xba*I/*Hin*dIII fragment from the plasmid pPX4019
containing the *pal* gene and the consensus ribosome
binding site from pET27b into the similarly digested
plasmid pBAD18-Cm (see Figure 1). Clones were screened
by restriction analysis followed by expression studies
on selected candidates. One of the pBAD18-Cm isolates
which expressed P6 protein was designated pPX4020.

Example 3
Expression of Lipidated rP6 from pPX4019 and pPX4020

Qualitative expression studies comparing
different isolates, plasmid constructs, *E.coli* host
strains and concentration of inducer (IPTG for pPX4019,
L-arabinose (Sigma Chemical Co., St. Louis, MO) for
pPX4020) were all performed in a similar manner to
provide for a consistent background for comparison.
Single colony isolates were grown overnight at 37°C in

HySoy™ media, 1% glucose and the appropriate antibiotic
for plasmid selection (pPX4019, 15 μg/ml Kanamycin; and
pPX4020, 15 μg/ml Chloramphenicol). These cultures
were diluted to a OD_{600}= 0.5 in HySoy™ 1% glycerol and
antibiotic, grown at 37°C to a OD_{600}= 2-4 and induced.
Samples equivalent to a OD_{600}= 1.0 were taken at time
points just prior to induction, at two hours and 18
hours post-induction. These samples were centrifuged
and the cell pellets resuspended in 150 μl SDS-PAGE
loading buffer (ISS, Natick, MA). Comparisons were
made from Coomassie blue stained 15% SDS-PAGE gels with
15μl of sample loaded per lane.

E. coli Host Cell Strains Used to Express Lipidated rP6
from Plasmid pPX4019:

DH5α - ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recAlendA1*
hsdR17(r_k^-,m_k^+) *phoA supE44*λ^- *thi-1 gyrA96 relA1* (Life
Technologies, Rockville, MD)

BL21(DE3) - *ompT lon hsdS*$_B$ (r_B^- m $_B^-$) *gal dcm* (DE3)
(Novagen)

E. coli Host Cell Strains Used to Express Lipidated rP6
from Plasmid pPX4020:

DH5α - ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recAlendA1*
hsdR17(r_k^-,m_k^+) *phoA supE44*λ^- *thi-1 gyrA96 relA1* (Life
Technologies)

BLR - *ompT lon hsdS*$_B$ (r_B^- m $_B^-$) *gal dcm* Δ(*srl-
recA*)306::Tn*10*(tet^r) (Novagen)

BL21(DE3) - *ompT lon hsdS*$_B$ (r_B^- m $_B^-$) *gal dcm* (DE3)
(Novagen)

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Growth of *E. coli* BLR(pPX4020) expressing L-rP6:

Recombinant *E. coli* cells expressing lipidated rP6 were grown in a fermenter as described below. Growth media containing the following materials was prepared and sterilized *in situ* for fermenters and by autoclaving for flask growth.

Solution A.

Material	g/L*
Potassium phosphate, monobasic	3.0
Potassium phosphate, dibasic	7.0
Ammonium sulfate	1.0
Sodium citrate dihydrate	1.0
Ferrous sulfate, heptahydrate	0.09
Glycerol (Remove flask medium before adding)	5 mL/L
Sodium Sulfate	0.58
1000X Trace Mineral Solution (see below)	1 mL/L
Do not pH adjust - Autoclave	

* unless otherwise stated

1000X Trace Mineral Solution	g/100 mL
Zinc sulfate, heptahydrate	3.0
Cupric sulfate, pentahydrate	0.9
Manganese sulfate, monohydrate	0.42
Cobalt chloride, hexahydrate	0.06
Molybdic acid	0.15

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Pre-inoculation Feed Solution: MCG

Ingredient	g/L
Glucose	500
Magnesium sulfate, heptahydrate	11
Calcium chloride	0.83

EDTA Solution

Ingredient	g/L
EDTA	186.15

Sterile media containing Solution A and the
Mineral solution was aliquoted into shake flasks and 20
ml of MCG added per liter of media. Chloramphenicol
was added to 50 μg/ml. The starter cultures were
inoculated with 200-300μl of E. coli BLR (pPX4020)
from a frozen stock. The cells were grown at 30°C with
aeration for 16 hours. A 10 liter fermenter containing
growth media, supplemented as above, was inoculated
with the above culture to an OD_{600} of approximately 0.2.
Fermenter control parameters were set as
follows:

Temperature	36°C
pH=	7.0 +/- 0.1
D.O.=	20 to 50% by agitation and pure oxygen
Air and oxygen flow =	20% on rotameter
Antifoam	one drop if necessary
Backpressure	0.5 bar

The following solutions were used for control
of run parameters and were sterilized prior to use:
PPG-2000—200 mL for foam control if necessary, 40%

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ammonium hydroxide—1 L for pH control during growth,
50% glucose—1 L to feed when pH began to rise due to
glucose depletion - for continued growth, 4 N acetic
acid—500 mL for pH control during induction, 25%
arabinose solution, (250g/L) sterile filtered + 1%
glycerol - 250 mL (pre-autoclaved). The
arabinose/glycerol solution was used to feed after
final glucose addition when pH began to rise due to
glucose depletion to induce production of protein. The
arabinose solution was filter sterilized prior to
addition of the autoclaved glycerol stock. The
solution was made by adding 100 mL glycerol and 400 mL
of 25% arabinose solution for a 10 L fermentation. The
final fermenter concentration contained 10 grams/liter
arabinose and 10 ml/Liter glycerol.

After inoculation of the fermenter, base
(sodium hydroxide) was added as needed for pH control,
along with anti-foam (PPG-2000) as needed. Pure oxygen
was fed at 800 rpms. When the pH rose above 7.0, 900
mL of 50% glucose was fed to the culture. When the pH
was greater than or equal to 7.1, an additional 200 mL
of 50% glucose solution was added. These conditions
were continued until an OD_{600} of approximately 50 was
reached.

After an OD_{600} of approximately 50 was
reached, when the pH rose above 7.0 again, 500 mL of
arabinose/glycerol solution was added to induce
expression of the LrP6. At this time, acid was used
for further pH control. Incubation was continued for
three hours post-induction, and then the culture was
harvested following addition of 10 mL per liter of 500
mM EDTA solution, pH 8. The culture broth was stored
at 4°C until purification of the lipidated rP6.

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Example 4
Batch Scale Analytical Purification of Lipidated rP6
by Differential Detergent Membrane Extraction

5   Batches of the lipidated rP6 expressed from
pPX4020 were utilized in subsequent experiments, and
were purified by differential detergent membrane
extraction as follows:

          1) Isolation of the *E.coli* membrane fraction:
10   Frozen bacterial cell pellets obtained from
fermentation were thawed and suspended in 10 mM HEPES-
NaOH, pH 7.4, 1 mM Na$_2$EDTA with a volume of buffer
equal to five times the weight of the frozen cell
pellet.   The cell suspension was homogenized in a
15   Microfluidics (Newton, MA) 110-Y microfluidizer to lyse
the cells.   The membranes were obtained from the cell
lysate by differential centrifugation (300,000 x g for
1 hour).   The membranes were washed twice with the same
volume of the buffer used for lysis, and then frozen as
20   a pellet.

          2) Solubilization of Lipidated rP6 from *E.*
coli membranes: The lipidated P6 was solubilized from
E.coli using differential detergent extraction similar
to that described by Zlotnick et al (33) and Green et
25   al (22).   All extractions were carried out for 30
minutes at room temperature unless otherwise stated.
All centrifugations were performed using a Beckman 45Ti
rotor at 42,000 rpm for one hour with the temperature
of the rotor controlled to 10°C unless stated
30   differently.   *E. coli* membranes were suspended in 10 mM
HEPES-NaOH, pH 7.4, 1mM MgCl$_2$ and extracted twice with
Triton™ X-100 (Calbiochem-Novabiochem International,
San Diego, CA) at a final concentration of 1% (w/v) to
remove inner membrane components.   The resulting outer
35   membrane pellet was suspended in 50mM Tris™ HCl, pH 8,

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5mM Na$_2$EDTA, which was also used to suspend the subsequent pellets prior to the solubilization of the lipidated rP6. The outer membranes were then sequentially extracted two times with 1% Zwittergent™ 3-14; two times with 1% Zwittergent™ 3-14 and 0.5M NaCl; two times with 1% N-lauryl sarcosine, Na salt; 1% Zwittergent™ 3-14. The final pellet obtained after these extractions was extracted with 0.2% Zwittergent™ 3-12 in 10mM Tris™ HCl, pH 8, 1mM Na$_2$EDTA for 45 minutes at 55°C with intermittent mixing followed by centrifugation for at least one hour. The supernatant from this final extraction contained the lipidated rP6.

      3) Purification of Lipidated rP6: The lipidated rP6-containing extract obtained as described above was further purified by anion exchange chromatography utilizing a DEAE fast flow resin (Amersham Pharmacia Biotech, Piscataway, NJ) and the same low ionic strength buffer utilized for extraction. The solubilized lipidated rP6 was adsorbed to a DEAE fast flow column with a bed volume of approximately 20mL. The column was developed with a 0-0.2M NaCl gradient over 40 minutes, followed by 20 minutes of an isocratic elution with 0.2M NaCl. The lipidated rP6 eluted during the isocratic phase of the development. Most of the other proteins remained adsorbed to the column and were later desorbed with 1M NaCl. Approximately 100mg was purified from 200g cells.

      4) Characterization of purified lipidated rP6 by SDS-PAGE and Western blot: The homogeneity of purified lipidated rP6 was assessed by SDS-PAGE in the Laemmli buffer system, followed by laser densitometry of the stained gel. Approximately 10µg of LrP6 was analyzed from both the crude extract and anion exchange purified pool on a 15% SDS-PAGE gel (53). The gel was stained with Coomassie Blue and scanned in a laser

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densitometer. Laser densitometry of the Coomassie
stained gel (Figure 2A, lane 2) revealed a single peak
of greater than 98% homogeneity in the pooled anion
exchange fractions, indicating that lipidated rP6 had
been purified to near homogeneity. The identity of
lipidated rP6 in these samples was verified by reacting
a western blot of the same samples used to determine
homogeneity with monoclonal antibodies specific for the
Haemophilus influenzae P6, which do not react to the
related protein of *E. coli* (data not shown). Results
of the western blot analysis are shown in Figure 2B.
The lipidated rP6 band was the only band reactive with
the P6-specific monoclonal antibody in either the crude
extract (lane 1) or the pooled fractions (lane 2).
This indicated that the purified protein is, in fact,
P6. No degradation products were observed.

Example 5
Large Scale Purification of Lipidated rP6 Using
Differential Detergent Membrane Extraction

The fermentation broth of *E. coli* cells
expressing lipidated rP6 was adjusted to 10 mM EDTA and
diluted to less than or equal to 10% wet weight
cells/volume prior to homogenization. The cells were
then lysed with a high-pressure microfluidizer and
diafiltered at room temperature with a sequence of
buffers using a cross-flow membrane filtration device.
It was determined that the minimum membrane area to
allow efficient mass transport of solubilized proteins
through the membrane was approximately 0.002 m^2/g wet
weight cells. The solubilized proteins of approximate
size less than the 1000 kD molecular weight cut-off
rating of the membrane passed through with the
permeate, while larger molecules and unsolubilized

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proteins were retained. The sequence of diafiltration
steps was as follows:

(1) The lysed fermentation broth was
diafiltered with 10 mM Hepes/1 mM EDTA/pH 8.0 (lysis
buffer) at a volume equal to three times the volume of
the retentate to remove intracellular and extracellular
contaminants through the permeate.

(2) The lysate was diafiltered three times
with 10 mM Hepes/1mM $MgCl_2$ /0.2% Triton™ X-100 to
solubilize and remove inner membrane proteins. The
Mg^{++} ions stabilized the outer membrane, therefore, the
outer membrane proteins were not solubilized in the
presence of Triton™ X-100.

(3) The lysate was diafiltered three times
with 50 mM Tris™ /5 mM EDTA/0.2% Zwittergent™ 3-14 to
solubilize and remove outer membrane proteins (but not
lipidated rP6). The EDTA serves to sequester the Mg^{++}
ions from step (2), as well as to prevent proteolysis.

(4) The lysate was diafiltered three times
with 50 mM Tris™/5 mM EDTA/0.5 M NaCl/0.2% Zwittergent™
3-14 to solubilize and remove additional proteins.
NaCl was added to the buffer in this step to disrupt
any ionic interactions between membrane proteins and
membranes. This step was performed because lipidated
rP6 is a PAL, and the salt serves to remove membrane-
bound proteins (but not lipidated rP6) from the
membrane/outer membrane protein complex. The
diafiltration was continued with three retentate
volumes of 50 mM Tris™/5mM EDTA to reduce the
Zwittergent™ concentration in the retentate.

(5) The lysate was diafiltered three times
with 50 mM Tris™ /5 mM EDTA/0.2% sarcosyl to remove
additional membrane bound proteins (but not lipidated
rP6) and then diafiltered three times with 50 mM
Tris™/5 mM EDTA to reduce the sarcosyl concentration in

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the retentate.

(6)   The lysate was diafiltered three times
with 10 mM phosphate/0.2% Zwittergent™ 3-12 to remove
additional membrane bound proteins (but not lipidated
rP6), and then diafiltered three times with 10 mM
sodium phosphate to reduce the Zwittergent™ 3-12
concentration in the retentate.

(7)   The lysate was concentrated to 20% of
its original volume and then diafiltered three times
with 10 mM sodium phosphate/0.2% Zwittergent™ 3-12 at
55°C to solubilize lipidated rP6, which was collected
through the permeate. The concentration step was
performed prior to diafiltration to increase the
concentration of lipidated rP6 in the permeate. The
diafiltration was continued for three additional
retentate volumes with 10 mM sodium phosphate at 55°C
to reduce the Zwittergent™ 3-12 concentration in the
retentate. This heating step was performed because (as
in step (4) above)) lipidated rP6 is a PAL, and heating
serves to remove lipidated rP6 from the
membrane/membrane protein complex. Finally, the
diafiltration was concluded with three retentate
volumes of 10 mM sodium phosphate at 55°C.

During the diafiltration steps, the
transmembrane pressure was maintained at approximately
10 psi and the cross flow rate was maintained at
approximately 120-180 lmh. All the diafiltration
processes were run at room temperature, except the
final 55°C extraction step, which was run at the higher
temperature to solubilize lipidated rP6. The permeate
flux ranged from 30 to 50 lmh, which was sufficiently
high for the extraction process to be practical and
scalable.

During the extraction, samples were taken at
various points for analysis by SDS-PAGE to evaluate the

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effect of various diafiltration steps on the extraction
of proteins. Samples were precipitated by alcohol
addition, centrifuged, and then resolubilized at 20% of
the original volume in SDS sample prep buffer. This
method of preparing samples concentrated the sample and
reduced the Triton™ X-100 or Zwittergent™ 3-12
concentration of the samples. Triton™ X-100 or
Zwittergent™ 3-12 interfere with the binding of SDS to
the sample and reduced the resolution of bands on gels.
Ten μl of each sample was loaded on to Novex 10%
acrylamide gels and the gels were run for 60-90
minutes at 125 Volts.

     A typical SDS-PAGE analysis of the samples
taken from the permeate streams during the extraction
process of lipidated rP6 is shown in Figures 2 and 3.
Lipidated rP6 ran at 15 kilodaltons (kD) on these gels.
The gels show that some contaminating proteins were
removed during diafiltration with lysis buffer and
buffer containing various detergents. There was very
little loss of lipidated rP6 during these diafiltration
steps. During the final Zwittergent™ 3-12
diafiltration step at 55°C, lipidated rP6 was extracted
in a partially purified state. At the end of the
second Zwittergent™ 3-12 diafiltration step at 55°C,
very little lipidated rP6 was present in the permeate
stream. This suggested that most of the solubilized
lipidated rP6 had been recovered through the permeate.
Other experiments have shown that very little lipidated
rP6 remained unsolubilized in the retentate after the
completion of the diafiltration process. The 15 kD
band of the Zwittergent™ 3-12 / 55°C extract was shown
to be lipidated rP6 by western analysis (data not
shown).

     The use of Zwittergent™ 3-12 in the
solubilization of lipidated rP6 resulted in an extract

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that contained several proteins in addition to
lipidated rP6. The homogeneity of this extract was
determined to be approximately 78% lipidated rP6
(Figure 2, Panel A, lane 1). While this is a high
5   degree of homogeneity for an initial solubilization, it
was desired to separate the LrP6 from these *E. coli*
proteins if possible. This was carried out as
described in Example 6.

Example 6
Further Purification Of Purified Lipidated rP6
by Anion Exchange Chromatography

Anion exchange chromatography was used to
further purify the lipidated rP6 described in Example
15   5, because it has been used successfully to purify the
non-lipidated rP6. The lipidated rP6 adsorbs more
tightly to the DEAE resin than the rP6, which typically
elutes with 0.1M NaCl. The lipidated rP6 in 0.2%
Zwittergent™ required 0.2M NaCl in the buffer before
20   desorbtion occurred. The *E. coli* proteins remained
adsorbed to the anion exchange resin (DEAE) until after
the lipidated rP6 was eluted.
        The homogeneity and identity of the lipidated
25   rP6 extracted and purified with 0.2% Zwittergent™ 3-12
are shown in Figure 2. The homogeneity of the purified
lipidated rP6 was determined to be greater than 98%
(Figure 2, Panel A, lane 2).

30   Example 7
Determination of Molecular Weight by
MALDI-TOF Mass Spectral Analysis:

Accurate measurement of the molecular weight
35   of lipidated rP6 expressed from pPX4020 with the

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arabinose inducible promoter was carried out by Matrix
Assisted Laser Desorption/Ionization Time-of-flight
(MALDI-TOF) mass spectrometry using a Finnigan Mat
Lasermat™ 2000 linear mass analyzer (Finnigan Mat,
Ltd., San Jose, CA). The Lasermat™ uses the technique
of matrix-assisted laser desorption (54) to ionize the
sample and Time of Flight to analyze the ions produced.
The sample was embedded in a matrix of 3,5-dimethoxy-4-
hydroxy-cinnamic acid (sinapinic acid) to enhance
ionization of the sample. One microliter of the sample
containing 5-10 pmol of the purified protein was mixed
with 1 μl of the matrix (10 mg/ml) dissolved in 70%
(v/v) aqueous acetonitrile containing 0.1% (v/v)
trifluoroacetic acid. One microliter of this sample
and matrix mixture was loaded on a sample slide,
allowed to dry and irradiated by a short pulse of UV
light from a laser. Protein samples usually generate a
relatively simple spectra in this method, since
protein-related ions produced are predominantly of
charge states z=+1 [M+H]$^+$ and z=+2 [M+2H]$^{2+}$. Cytochrome
C from bovine heart (Sigma Chemical Co., St. Louis, MO)
of molecular weight 12,230.9 was used for external
calibration.

The molecular weight of lipidated rP6 in the
sample used was determined to be 15,078. In addition
to the expected [M+H]$^+$ molecular ion, the [M+2H]$^{2+}$
molecular ion of lipidated rP6 was also observed. The
theoretical molecular weight of P6 containing a
tripalmitoyl cysteine residue at its N-terminus is
15,024 and the predicted molecular weight of P6
unprocessed by signal peptidase II is 16,016.66,
whereas the predicted molecular weight of unlipidated
P6 cleaved by signal peptidase II is 14,234.66. Thus,
these results are consistent with the expression of the
lipidated form of rP6 by *E. coli*.

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Example 8
Amino Acid Composition Analysis

A sample of lipidated rP6 for amino acid analysis was dried down in glass tubes, followed by hydrolysis using 100 μl of 6 N HCl containing 5% phenol and 1% 2-mercaptoethanol under vacuum for 22 hours at 110°C. The samples were subsequently dried under vacuum, followed by resolubilization in the sample dilution buffer Na-S (Beckman Instruments, Inc., Fullerton, CA). The amino acid composition was determined on a Beckman model 6300 Amino Acid Analyzer (55) using a three step Na-citrate gradient according to manufacturer's instructions. Threonine and serine residues were not corrected for destruction. Since cysteine and tryptophan residues were not determined by the method used, the results were expressed as mol of residues per mol of lipidated rP6 based on the theoretical molecular weight of unlipidated rP6 minus cysteines, which equals 14,132.4 (lipidated rP6 does not contain Trp). The results are shown in Table 1 and represent the mean of duplicate determinations. The results are consistent with the signal sequence of the *pal* gene having been removed by *E. coli*.

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Table 1

Amino Acid Analysis of lipidated rP6

Amino acid	Experimental mol residues/mol	Theoretical Mature mol residues/mol	Theoretical Pro-peptide mol residues/mol
Asp + Asn	16.6	17	18
Thr	6.6	7	7
Ser	7.0	6	8
Glu + Gln	12.5	12	12
Pro	2.8	3	3
Gly	16.9	16	17
Ala	18.9	21	26
Val	10.5	10	13
Met	0.2	0	1
Ile	2.9	3	3
Leu	8.4	9	12
Tyr	10.1	11	11
Phe	3.1	3	4
His	3.1	2	2
Lys	6.6	7	9
Arg	6.5	6	6
Cys	ND	1	1
Trp	ND	0	0

ND = Not determined

Example 9

Amino-Terminal Amino Acid Sequence Analysis

Amino-terminal protein sequence analysis was
carried out using an Applied Biosystems Model 477A
Protein/Peptide Sequencer equipped with an on-line
Model 120A PTH Analyzer (Applied Biosystems, Foster

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City, CA). After the cleavage of each successive
amino-terminus, the anilinothiazolinone derivative
formed was converted to the more stable
phenylthiohydantion (PTH) derivative by treatment with
25% trifluoroacetic acid at 64°C for 20 minutes. The
PTH derivatives were separated and identified on the
PTH analyzer by reversed-phase HPLC using an Brownlee
PTH C-18 column (particle size 5 μm, 2.1 mm i.d. x 22
cm l.; Applied Biosystems) with a modified two solvent
gradient system developed by the manufacturer (56).

When lipidated rP6 (400 pmoles) was subjected
to amino-terminal amino acid sequence analysis, no
sequence data could be obtained. This suggested that
the primary (or a secondary) amino group of the amino-
terminal amino acid was not available for the
sequencing chemistry, i.e., the amino-terminal residue
of LrP6 was blocked. In order to substantiate that the
inability to generate sequence data was not due to any
instrument malfunction, a control experiment was
subsequently run in which a mixture of 400 pmoles of
lipidated rP6 and 200 pmoles of beta-lactoglobulin was
subjected to amino-terminal sequence analysis. A
single sequence representing the amino-terminal
sequence of beta-lactoglobulin was obtained, which
confirmed that the amino-terminal residue of lipidated
rP6 was essentially blocked.

Example 10
Immunogenicity of Lipidated rP6
Compared to Non-lipidated rP6

The relative immunogenicity of the purified
lipidated recombinant P6 and non-lipidated recombinant
P6 (28) were compared in Swiss-Webster mice. Each five
μg dose of each protein was mixed with 100 μg AlPO$_4$ and

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50 μg 3-O-deacylated monophosphoryl lipid A (MPL™)
(Ribi Immunochemicals, Hamilton, MT) were used to
immunize mice subcutaneously at weeks 0, 4, and 6.
Blood samples were taken at weeks 0, 4, 6, and 8.
Other groups of mice were immunized with mixtures of
either non-lipidated rP6 or lipidated rP6 and the UspA2
protein of *Moraxella catarrhalis* (49), a causative
agent of bacterial otitis media, and recombinant
lipidated rP4 (50). These mixtures were also
adjuvanted with AlPO$_4$ and MPL™ as above.

Antisera obtained from the mice were analyzed
by ELISA for antibodies against either the P6, P4, or
UspA2 proteins. ELISA titers were determined (22,28)
for either pooled sera or individual animals and then
the geometric mean titer (GMT) derived. The results
are shown in Tables 2 and 3.

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Table 2
Anti-P6 ELISA Titers

| | | Anti-P6 ELISA Titer: | | |
Immunogen		Week 0	Week 6	Week 8
5 μg rP6 (non-lipidated),	GMT		1,369	17,686
	Pool	<50	40,715	147,985
5 μg rP6 (lipidated)	GMT		341,987	780,179
	Pool	<50	706,826	786,917
5 μg each rP6 (non-lipidated), rP4, UspA	GMT		425	15,015
	Pool	<50	692	64,913
5 μg each rP6 (lipidated), rP4, UspA	GMT		251,731	1,052,527
	Pool	<50	739,896	1,268,527

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Table 3
Anti-P4 and UspA2 ELISA Titers

Immunogen		Anti-P4 ELISA Titer:		Anti-UspA2 ELISA Titer:	
		Week 0	Week 4	Week 0	Week 4
5 μg each rP6 (non-lipidated),	GMT		84,677		143,285
rP4, UspA	Pool	<50	189,980	<50	247,003
5 μg each rP6 (lipidated),	GMT		136,412		257,751
rP4, UspA	Pool	<50	197,361	<50	335,548

The lipidated rP6 is at least one log more immunogenic than the non-lipidated rP6 when administered alone with MPL™ and AlPO$_4$ adjuvants. When combined with rP4 and UspA2, no antigenic competition was observed. In fact, the response to the lipidated rP6 was increased to approximately 1.5 logs greater than the response to the non-lipidated rP6.

Anaylsis of the immune response to the UspA2 and rP4 antigens shows that the addition of the lipidated rP6 did not alter the immune response to these antigens as compared to addition of the non-lipidated rP6. Neither antigen had any effect on the normal immune response seen when lipidated rP4 and UspA2 were mixed together. This demonstrated the compatability of these antigens.

Example 11
Bactericidal Activity of Mouse Antisera

Biologic activity of the antisera directed against the lipidated rP6 and the lipidated rP6/rP4 mixtures was demonstrated using an *in vitro* bactericidal assay. This assay was performed as previously described (22,28) using nontypable *H. influenzae* strain P861454 as the target. The results are shown in Table 4:

Table 4
In Vitro Bactericidal Activity
Of Antisera From Tables 2 and 3

Immunogen:	Week 6 Sera		Week 8 Sera	
	BC Titer	Times (X) Background	BC Titer	Times (X) Background
rP6	3,200	8X	12,800	16X
L-rP6	3,200	4X	12,800	16X
rP6, rP4, UspA2	3,200	4X	6,400	8X
L-rP6, rP4, UspA2	3,200	4X	12,800	16X

The results demonstrated that the lipidated rP6 elicited biologically active antibodies in this assay. While the absolute titers did not differ between the lipidated and non-lipidated antisera, this may be due to the antisera being maximally bactericidal in this assay system, especially since the preimmune

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sera demonstrated a high degree of nonspecific killing
with the complement source used in this assay. The
lipidated rP6/rP4 mixture also elicited bactericidal
antibodies at titers equivalent to those obtained with
the non-lipidated rP6/rP4 mixture. It was not possible
to distinguish between the bactericidal activity of the
anti-rP4 antibodies and the anti-rP6 antibodies in this
assay, but it is clear that the mixture of the
Haemophilus antigens elicited highly bactericidal
antisera against nontypable H. influenzae.

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